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14. ABSTRACT

We hypothesize that the biomechanical perturbations of the brain that yield blast-induced mTBI in injured warfighters can be recreated with reasonable fidelity in rats under carefully controlled experimental conditions, and that several of the characteristic sequelae of blast-induced mTBI observed clinically can be reproduced in a rodent injury model. In many, if not most circumstances yielding blast mTBI, brain injury results from a combination of blast overpressure (BOP) (i.e. primary blast) and head acceleration and/or impact (i.e. tertiary blast). The mTBI resulting from these combined insults may be fundamentally different from that seen from either insult alone.

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INTRODUCTION

Many warfighters who sustain blast-induced TBI in combat are exposed to a brain insult resulting from a combination of both a shock wave and biomechanical perturbation related to rapid acceleration and/or impact with a solid object (MacDonald et al., 2011). The TBI resulting from these combined insults is likely to be fundamentally different from that seen from either insult alone. We hypothesize that the combined biomechanical perturbations of the brain that yield blast-induced mild TBI in injured warfighters can be recreated with reasonable fidelity in rats under carefully controlled experimental conditions, and that several of the characteristic sequelae of blast-induced mild TBI observed clinically can be reproduced in an established rodent injury model. We anticipate that this model can provide a valuable experimental tool to assist ongoing efforts to mitigate the risks and consequences of blast-induced mTBI in warfighters.

OVERALL PROJECT SUMMARY

Research accomplishments associated with each task outlined in the approved Statement of Work are described below.

Task 1

Manipulate and monitor blast exposure conditions (i.e. incident flow conditions) in the compression-driven shock tube and recreate with reasonable fidelity the biomechanical loading conditions estimated to underlie primary blast-induced mild TBI in warfighters. Establish a mild injury severity based upon loss of consciousness (LOC), histopathology, and neurological and neurobehavioral outcomes.

Task 1 progress: Blast exposure conditions in the cylindrical shock tube have been generally standardized to maximize fidelity and reproducibility, and pressure gauges in the rat holder record the ambient flow conditions during BOP exposure. We continue to secure rats 2.5 ft within the mouth of the tube in tautly drawn coarse mesh netting to minimize movement and reduce variability. Rotarod procedures have been shown to be an additional means to sensitively discern neurological/neurobehavioral disruptions resulting from mild TBI, and rotarod testing has been added to our battery of neurobehavioral evaluations which also include rotary pole, open field, and Morris water maze. Exposure to a single BOP alone does not produce robust neurobehavioral disruptions; discernable disruptions are only evident when BOP is combined with weight drop (task 3) or when rats are exposed to closely coupled repeated BOP. Visual discrimination procedures, in which rats are trained to distinguish and respond to visual cues by pressing left or right levers for delivery of food pellets have shown promise as a sensitive neurobehavioral assessment. Unfortunately, the training and testing requirements for visual discrimination assessments greatly restrict experimental subject through-put. We continue to complement light microscopic histopathological

assessments with high resolution ex vivo diffusion tensor imaging through a collaboration with investigators at the Center for In Vivo Microscopy at Duke University Medical Center. As described in a manuscript featured on the cover of Journal of Neurotrauma, these brain images revealed significant quantitative changes following closely coupled repeated BOP exposures, but not single exposures. DTI provides powerful comprehensive, quantitative analysis of sometimes subtle neuropathological changes throughout the entire brain, which is not possible with standard histopathological techniques.

Task 2

Establish conditions yielding a mild injury severity with a surgery-free adaptation of the weight drop brain injury model (or alternative) to create tertiary blast brain injury based upon LOC, histopathology, and neurological and neurobehavioral outcomes.

Task 2 progress: As previously described, we adapted the Marmarou weight drop technique to create tertiary (i.e. impact-acceleration) injury, and have characterized injuries resulting from a 500 g weight dropped from varied heights onto stainless steel discs affixed to the skulls of anesthetized rats resting on a foam pad. Injuries and neurobehavioral disruptions produced with this approach appear to be greater and more consistent than those produced using a removable headpiece placed over the scalp. Consequently, we have worked with this approach to combine BOP with weight drop with a minimal time separation (< 1 min) for task 3. Neurobehavioral disruptions and brain histopathological consequences of these weight drop exposures have been thoroughly characterized over a range of drop heights and now include rotarod performance as an outcome assessment. In addition, proteomic and genomic assessments have been performed to provide insights into the neurobiological underpinnings of these brain injuries.

Task 3

Combine BOP and the selected impact acceleration insult at multiple combined severities, and evaluate the histopathological, physiological, and neurobehavioral outcomes relative to those seen following each insult alone. Establish combined injury conditions to produce mTBI.

Task 3 progress: Combinations of BOP and weight drops of varied heights were continued using discs affixed to the skull before BOP exposure. In addition, injuries produced by these combined insults were compared to those generated by exposures to closely coupled repeated blasts (separated by 1 min). With either combination, neurobehavioral deficits were recorded that were greater and more persistent than produced by a single insult alone. These findings are consistent with the primary hypothesis of the project, namely that the TBI resulting from these combined insults is fundamentally different from that seen from either insult alone. As briefly summarized in

the appendix, these functional assessments were accompanied by a variety of neurochemical measurements, histopathological evaluations, EEG recordings, and novel imaging comparisons. In addition to providing a comprehensive characterization of the injury, it is anticipated that these varied analyses following single and combined insults will shed light on the means by which these insults might interact, underlying neurobiological mechanisms, and potential targets for countermeasures and therapeutic interventions. In general, these measurements have revealed several generally consistent neuropathological changes, proteomic and neurochemical changes, and altered outcomes following combined insults that were not apparent (or as large) following either insult alone.

Task 4

Using a mach stem wedge equipped with a high velocity piston impactor, instantaneously combine impact acceleration with BOP within the shock tube to produce and evaluate the concomitant combined effects of primary and tertiary blast relative to those seen following each insult alone. Establish a mild injury severity based upon loss of consciousness (LOC), histopathology, and neurological and neurobehavioral outcomes.

Task 4 progress: A new shock tube (advanced blast simulator) was designed and was delivered in November 2013 to accommodate this task. We await delivery of a rat holder incorporating a pneumatically driven piston that will enable near instantaneous blast and impact to be combined for improved fidelity of combined insults.

KEY RESEARCH ACCOMPLISHMENTS

Bulleted list of key research accomplishments emanating from this research.

- Shock tube BOP exposure conditions have been fully characterized and refined to yield a reproducible high fidelity simulation of blast TBI. In addition to the shockwave amplitude, duration, and impulse, the influence of positioning, holder orientation and subject immobilization on responses to blast waves in the shock tube have been recognized and addressed. To reduce confusion and “bad data” in the rapidly emerging preclinical literature, we have share our observations with a number of other institutions and laboratories in an attempt to define a standardized platform for high fidelity blast exposures and assessments so that interpretable results can be generated and meaningfully compared across laboratories.
- Neurobehavioral, neuropathological, and neurochemical consequences of shock tube BOP exposures of varied intensities have been comprehensively evaluated.
- Neurobehavioral, neuropathological, and neurochemical consequences of weight drop-induced impact acceleration of varied intensities, alone and in combination

with shock tube BOP exposures, have been comprehensively evaluated using a progression of weight drop approaches.

- Although the changes are subtle, algorithms have been developed to analyze EEG recordings through 30 days post-injury to distinguish electrophysiological consequences of individual and combined blast- and weight drop-induced brain insults.

REPORTABLE OUTCOMES

Reportable outcomes that have resulted from this research include the following.

Manuscripts

Kamnaksh A, Budde MD, Kovesdi E, Long JB, Frank JA, Agoston DV. Diffusion tensor imaging reveals acute subcortical changes after mild blast-induced traumatic brain injury. Sci Rep. 2014 May 2;4:4809. doi: 10.1038/srep04809.

Valiyaveettil M, Alamneh Y, Wang Y, Arun P, Oguntayo S, Wei Y, Long JB, Nambiar MP. Cytoskeletal protein alpha-II spectrin degradation in the brain of repeated blast exposed mice. Brain Res.. 2014 Feb 26;1549:32-41.

Calabrese E, Du F, Garman RH, Johnson GA, Riccio C, Tong LC, Long JB. Diffusion tensor imaging reveals white matter injury in a rat model of repetitive blast-induced traumatic brain injury. J Neurotrauma. 2014 May 15;31(10):938-50. doi: 10.1089/neu.2013.3144. Epub 2014 Mar 27.

Wang Y, Arun P, Wei Y, Oguntayo S, Gharavi RB, Valiyaveettil M, Nambiar MP, Long JB. Repeated Blast Exposures cause DNA Fragmentation in Mice. J Neurotrauma. 2014 Mar 1;31(5):498-504 doi: 10.1089/neu.2013.3074.

Ahmed FA, Kamnaksh A, Kovesdi E, Long JB, Agoston DV. Long-term consequences of single and multiple mild blast exposure on select physiological parameters and blood-based biomarkers. Electrophoresis. 2013 Aug;34(15):2229-33. doi: 10.1002/elps.201300077. Epub 2013 Jul 8.

Arun P, Abu-Taleb R, Oguntayo S, Wang Y, Valiyaveettil M, Long JB, Nambiar MP. Acute mitochondrial dysfunction after blast exposure: potential role of mitochondrial glutamate oxaloacetate transaminase. J Neurotrauma. 2013 Oct 1;30(19):1645-51. doi: 10.1089/neu.2012.2834. Epub 2013 Aug 9.

Abstracts and meeting presentations

Y. Wang, Y Wei, L. Tang, Oguntayo, A. Edwards, C. Riccio, I. Gist, P. Arun, Van Albert and J. Long. Comparison of Combined Primary and Tertiary Blast Traumatic Brain Injury in Young and Middle Age Rats. Military Health Sciences Research Symposium (MHSRS), Fort Lauderdale, FL, August 2013.

P. Arun, R. Abu-Taleb, S. Oguntayo, A. Edwards, C. Riccio, S. VanAlbert, I. Gist, Y. Wang, M.P. Nambiar, J.B. Long. Tissue non-specific alkaline phosphatase in the etiology and diagnosis of tauopathy and chronic traumatic encephalopathy. Military Health System Research Symposium held at Fort Lauderdale, FL, August 2013.

P. Arun, R. Abu-Taleb, S. Oguntayo, A. Edwards, C. Riccio, S. VanAlbert, I. Gist, Y. Wang, M.P. Nambiar, J.B. Long. Tissue non-specific alkaline phosphatase in the etiology of tauopathy and chronic traumatic encephalopathy after traumatic brain injury. USUHS Research Day held at Bethesda, MD, May 2013.

Oral Presentation

P. Arun, Role of tissue non-specific alkaline phosphatase in the etiology of tauopathy and chronic traumatic encephalopathy. National Capital Region Traumatic Brain Injury Research Symposium held at National Institutes of Health, Bethesda, MD, April 2013.

Long, J.B. Shock Tube Simulations of Blast – Experience from WRAIR. Invited lecture to CNRM at USUHS, Nov, 2013.

Research proposals

Based upon work supported by this award, funding was sought through 14 research preproposals and proposals submitted to the CDMRP, DMRP, and MRMC BAA during this reporting period.

CONCLUSION

Results to date are consistent with the hypothesis that BOP generates a closely-associated insult to the brain (and other organs as well) and interactively compromises the brain's resilience and exacerbates the pathophysiological effects of other injury modalities such as impact acceleration (i.e. tertiary injury). With continued refinement, under carefully controlled experimental conditions the combined biomechanical perturbations of the brain that yield blast-induced mild TBI in injured warfighters can be recreated with reasonable fidelity to reproduce characteristic sequelae of blast-induced mild TBI. The endproduct model will provide an invaluable tool to define underlying neurobiological mechanisms and rationally establish effective countermeasures to lessen short-term impairments (e.g. return-to-duty) as well as chronic debilitation (e.g. chronic traumatic encephalopathy).

REFERENCES

Mac Donald CL, Johnson AM, Cooper D, Nelson EC, Werner NJ, Shimony JS, Snyder AZ, Raichle ME, Witherow JR, Fang R, Flaherty SF, Brody DL. Detection

of blast-related traumatic brain injury in U.S. military personnel. N Engl J Med. 2011 Jun 2;364(22):2091-100.

Marmarou A, Foda MA, van den Brink W, Campbell J, Kita H, Demetriadou K. A new model of diffuse brain injury in rats. Part I: Pathophysiology and biomechanics. J Neurosurg. 1994 Feb;80(2):291-300.

APPENDICES

Supporting data and published manuscripts attached.

Supporting Data

We explored various ways to combine BOP with weight drop injuries and concluded that affixing stainless steel discs to the skull prior to BOP exposure allowed weight drop to be performed within 30-45 sec of BOP exposure with more consistent and substantial injuries than were produced using a light weight Mylar headpiece for a surgery-free weight drop. Prompted by concern that the stainless steel disc might disrupt BOP effects on the brain, we recorded and compared ICP responses to BOP with and without a stainless steel disc in place. As seen in fig 1, ICP recordings were essentially identical with and without the stainless steel disc, and in both situations the ICP closely paralleled ambient static pressure recordings. Consequently, we concluded that the stainless steel disc does not fundamentally alter the effects on BOP on the brain in the shock tube. In addition, pressure recordings additionally revealed a substantial increase in ICP as a result of weight drop (fig 2).

Rotarod tests were demonstrated to provide a sensitive means to distinguish neurobehavioral disruptions following both BOP and weight drop and as shown in fig 3, closely coupled repeated BOP. Closely coupled repeated blast also disrupts performance in the open field and on the rotary pole (fig 3). Active avoidance was less sensitive to disruption by these combined insults (not shown).

Fig 1. ICP effects of BOP with (bottom) and without (top) stainless steel disc affixed to skull.

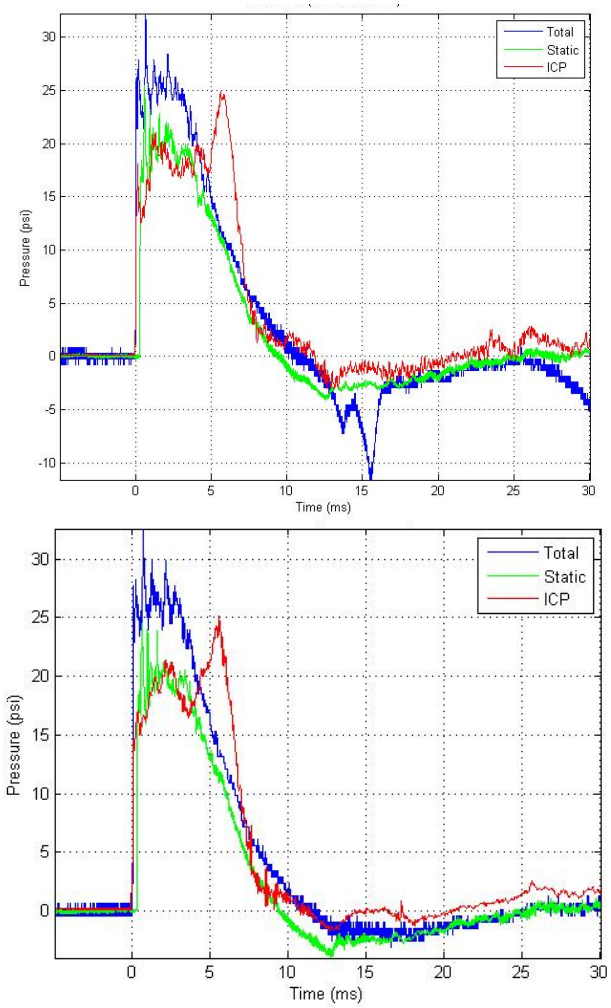


Fig 2. ICP response to weight drop (500 g, 125 cm).

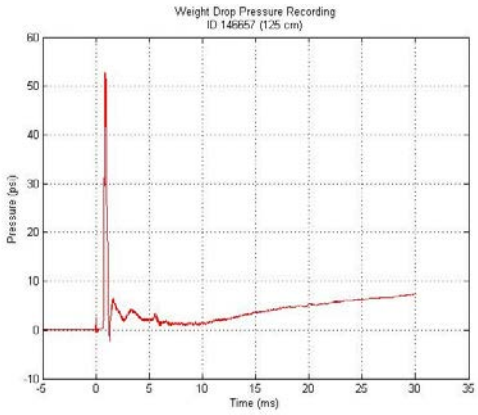
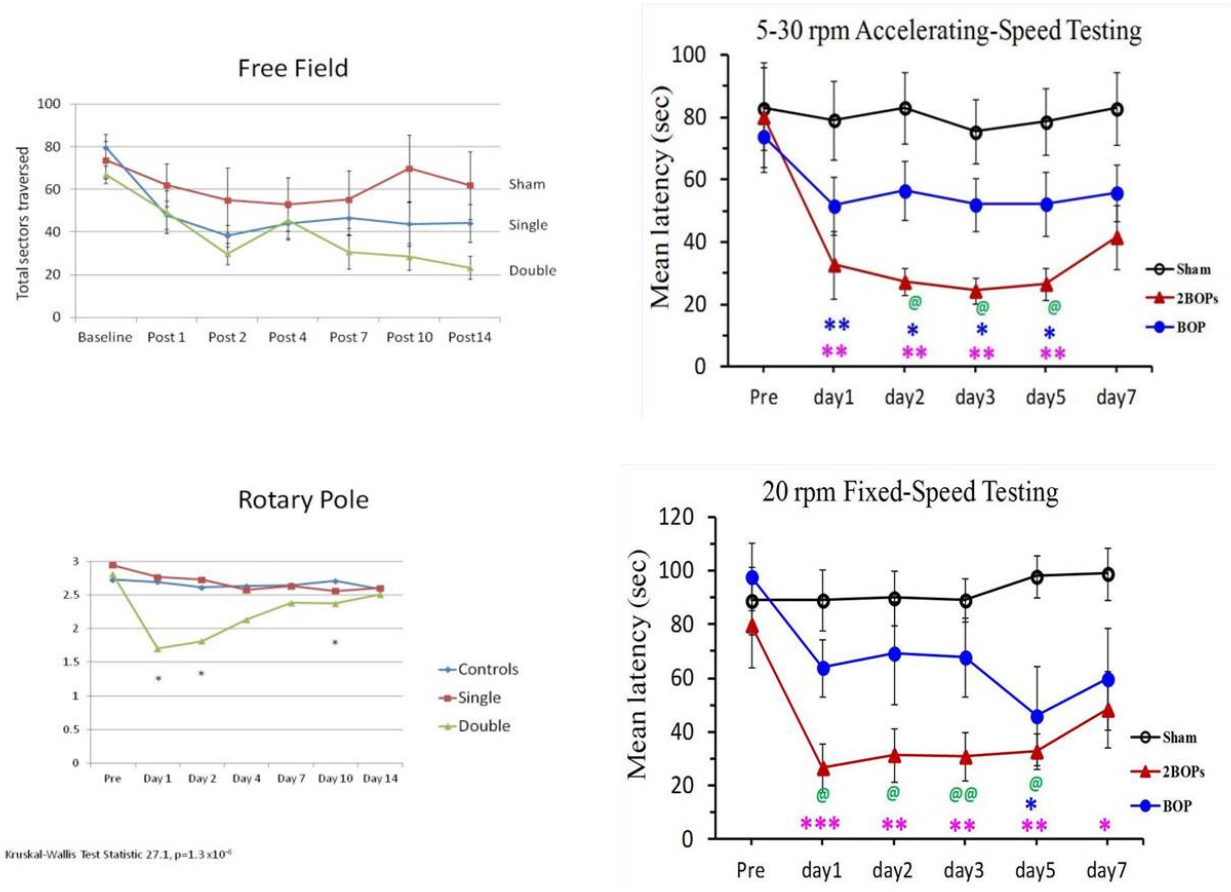


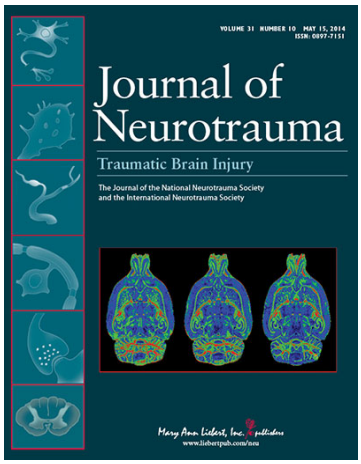
Fig 4. Neurobehavioral disruptions following closely coupled repeated blasts.



In addition to light microscopic characterization of neuropathological changes, brains were prepared for ex vivo imaging by collaborators at the Center for In Vivo Microscopy at the Duke University School of Medicine. Voxelwise analysis of diffusion tensor imaging (DTI) revealed consistent patterns of white matter injury in brains of rats exposed to closely coupled repeated blasts in contrast to a single blast exposure. Brains from the latter group were not appreciably affected and did not differ from those of sham injured rats. These findings, which were featured as a cover article in the May 15, 2014 issue of the Journal of Neurotrauma , show a significant increase in microstructural damage with a second blast exposure, suggesting that primary bTBI may sensitize the brain to subsequent injury.

These findings support the hypothesized interplay of closely coupled combined insults to the brain (in this case with BOP followed by BOP rather than

Fig 5. DTI reveals microstructural damage with repeated BOP



impact/acceleration).

Chronic traumatic encephalopathy (CTE) is a Tau protein-linked neurodegenerative disorder in which hyperphosphorylation of Tau protein is thought to disrupt microtubule assembly in neurons and potentially lead to the formation of neurofibrillary tangles. Dephosphorylation of pTau is critical to prevent tauopathy and to restore disrupted microtubule assembly and is primarily accomplished by the enzyme tissue non-specific alkaline phosphatase (TNAP). Decrease in its activity can lead to accumulation of pTau, tauopathy and CTE. We have observed regionally selective increases in pTau in brains of rats following blast and weight drop injuries which are accompanied by significantly decreased TNAP activity (figs 6-8). In addition to playing a potentially important role in the etiology of tauopathy and CTE, decreased activity of alkaline phosphatase in the plasma (fig 9) points to the potential utility of this enzyme as a biomarker of taupathy/CTE.

Under these injury conditions, seizures and epileptiform

Fig 6.

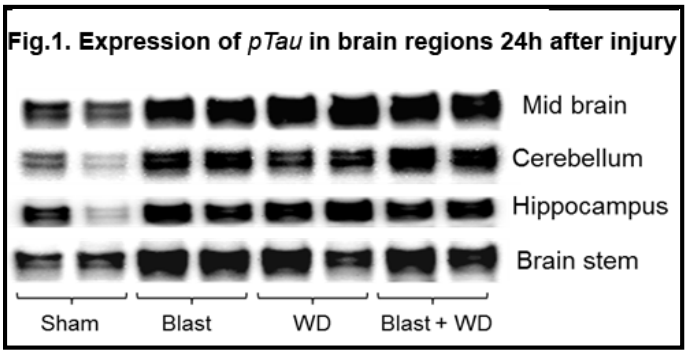


Fig 7.

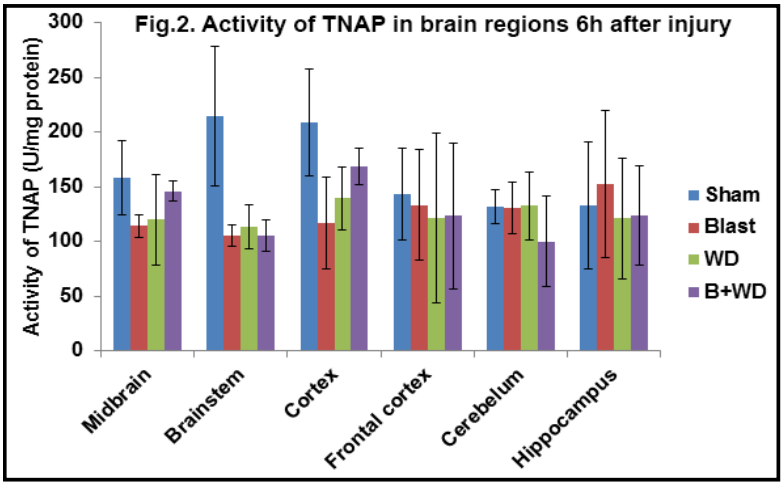
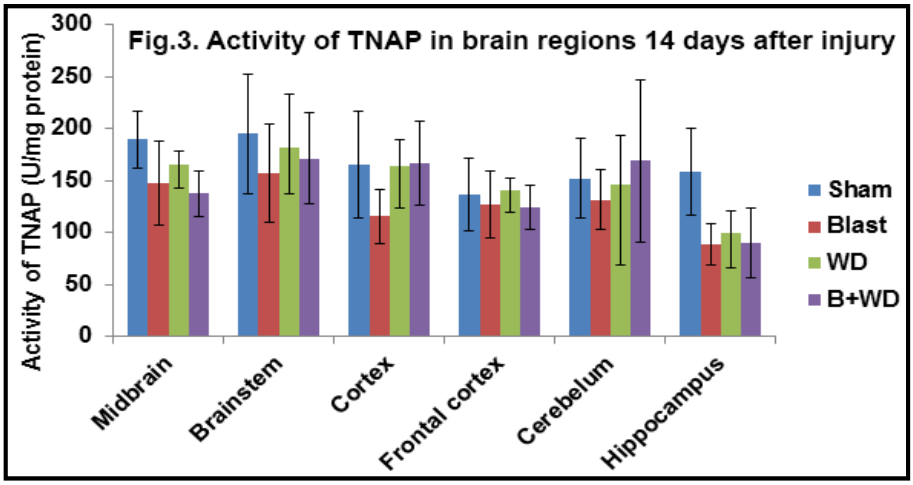
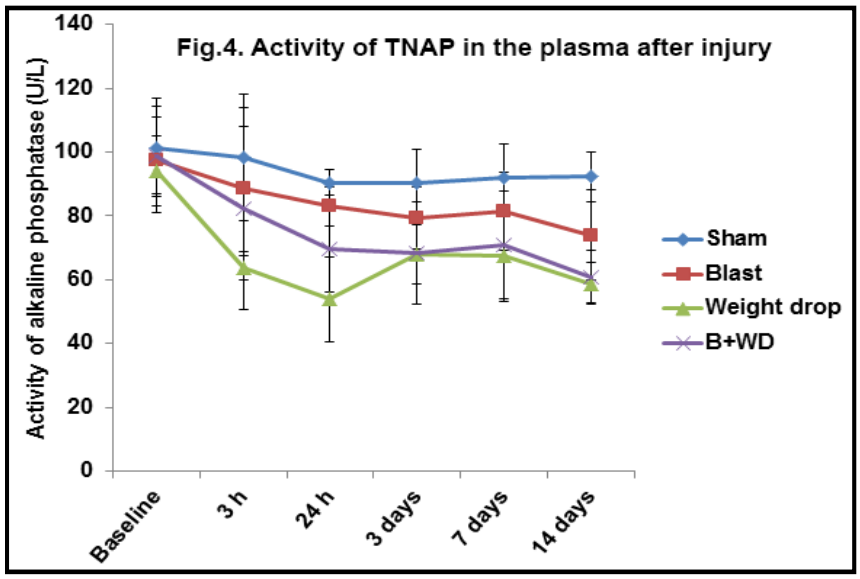


Fig 8.



discharges have proven to be sparse and are not useful to identify injury severity and monitor further deterioration and/or recovery
Consequently, we are exploring delta wave power density fluctuations for analysis.

Fig 9.



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Short Communication

Long-term consequences of single and multiple mild blast exposure on select physiological parameters and blood-based biomarkers

Mild traumatic brain injury (mTBI), especially when it is repeated (rmTBI), can lead to progressive degenerative diseases and lasting neuropsychiatric abnormalities. To better understand the long-term pathobiological changes in mTBI and rmTBI, we exposed rats to single or repeated (5 total; administered on consecutive days) mild blast overpressure, monitored changes in physiological parameters, and determined the plasma levels of select biomarkers at 42 days post injury by proteomics. We unexpectedly found comparable changes in arterial oxygen saturation levels and heart rates of single-injured (SI) and multiple-injured (MI) rats throughout the observation period. Our analyses indicated lasting oxidative stress, vascular abnormalities, and neuronal and glial cell loss in both injured groups. However, MI rats exhibited a relatively more pronounced increase in the plasma levels of most of the tested markers—particularly those associated with inflammation—albeit the differences between the two injured groups were not statistically significant. Our findings indicate that the frequency of blast exposures is an important determinant of the resulting cumulative damage in rmTBI.

Keywords:
Animal models / Brain trauma / Experimental / Physiology / Proteomics
DOI 10.1002/elps.201300077

Mild traumatic brain injury (mTBI) accounts for the majority of civilian and military traumatic brain injury (TBI) cases [1, 2]. In both civilian and military environments, affected individuals (e.g. football players) often sustain additional mild injuries. mTBI symptoms are typically mild and transient, however, repeated mild TBIs (rmTBI) can result in disproportionately severe acute symptoms suggesting some sort of cumulative effect of repeated injuries [3]. rmTBIs also increase the risk of developing late onset, progressive degenerative conditions such as chronic traumatic encephalopathy [4]. Despite their high prevalence, the pathobiology and consequently the diagnosis and treatment of mTBI and rmTBI have not been adequately addressed.

In a previous study assessing some of the neurobehavioral, cellular, and molecular consequences of single and multiple mild blast exposure at an early (~2 h) and a later post injury time point (22 days), we unexpectedly found a mild cumulative effect following repeated injury [5]. Based on these findings, we hypothesized that the cumulative effect in rmTBI requires a longer post injury time period to manifest. To test this hypothesis, we extended our experimental timeline to 42 days post injury and utilized noninvasive, clinically relevant tools to follow long-term changes in basic physiological parameters and blood-based biomarkers.

A total of 30 Sprague Dawley male rats, weighing 245–265 g at arrival (Charles River Laboratories, Wilmington, MA, USA), were used in our study. Housing, handling, and experimental manipulations of animals have been described earlier [5]. After an acclimation and handling period of five days, animals were randomly assigned to the following groups: naïve ($N = 3$), single sham (SS; $N = 6$), single-injured (SI; $N = 7$), multiple sham (MS; $N = 6$), and multiple-injured (MI; $N = 8$). Naïve rats were kept in the Uniformed Services University (USU) animal facility for the duration of the study without any manipulation. SS rats were transported once from USU to Walter Reed Army Institute of Research (Silver Spring, MD, USA) and anesthetized in an induction chamber for

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Abbreviations: CCR5, chemokine (C-C motif) receptor 5; **FPR1**, formyl peptide receptor 1; **GFAP**, glial fibrillary acidic protein; **HIF-1α**, hypoxia-inducible factor-1α; **HNE**, 4-hydroxynonenal; **MBP**, myelin basic protein; **MI**, multiple-injured; **MMP8**, matrix metalloproteinase 8; **MS**, multiple sham; **mTBI**, mild TBI; **NF-H**, neurofilament-heavy chain; **p38**, p38 mitogen-activated protein kinase; **rmTBI**, repeated mild TBI; **SI**, single-injured; **SS**, single sham; **TBI**, traumatic brain injury; **TLR9**, Toll-like receptor 9; **USU**, Uniformed Services University; **VEGF**, vascular endothelial growth factor; **vWF**, von Willebrand factor

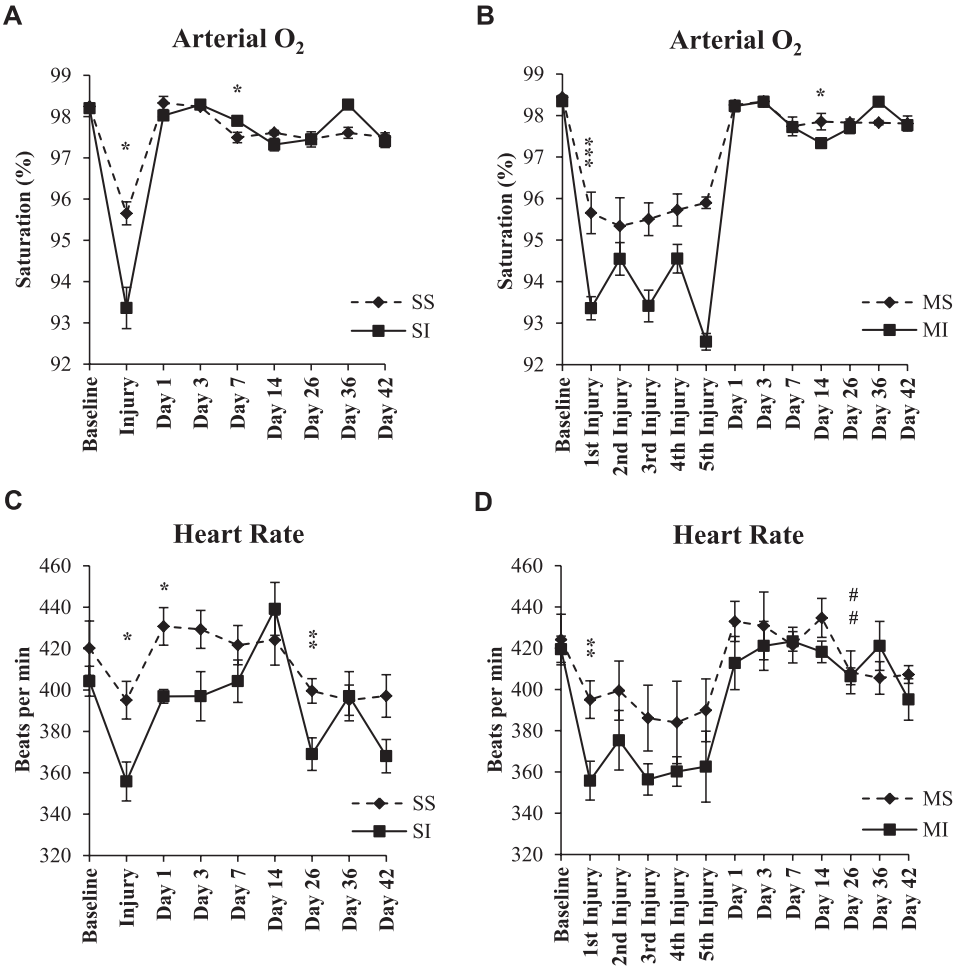

Figure 1. Arterial oxygen saturation levels (%) (A and B) and heart rates (beats per min) (C and D) of SS, SI, MS and MI rats. Measurements were obtained under isoflurane anesthesia at baseline, immediately following injury (5× for MI rats), and at days 1, 3, 7, 14, 26, 36, and 42 post injury. Data are presented as the mean ± SEM (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ sham versus injured; ##$p < 0.01$ SI versus MI).

6 min with 4% isoflurane (Forane; Baxter Healthcare Corporation, Deerfield, IL, USA). MS rats were similarly transported and anesthetized once per day for 5 consecutive days. SI and MI rats, weighing 300–330 g on injury day, underwent the same pre-injury procedures as their respective sham groups. SI and MI rats were then transferred to a compressed air-driven shock tube and exposed to a single or repeated (5 total administered on consecutive days) mild blast overpressure (average peak total pressure: ∼138 kPa) as described in detail [6, 7]. Mortality was: SI = 3 and MI = 4. Following the exposure(s), animals were transported back to the USU animal facility.

Arterial blood oxygen saturation (%), heart rate (beats per min), pulse distention (μm), and breath rate (breaths per min) were noninvasively monitored under light isoflurane anesthesia prior to injury (baseline), immediately after blast (or sham) exposure, and at days 1, 3, 7, 14, 26, 36, and 42 post injury using the MouseOx® Pulse Oximeter adopted for rats (Starr Life Sciences, Oakmont, PA, USA) [8]. At the termination of the experiment (day 42 post injury), rats (Naïve = 3, SS = 6, SI = 4, MS = 6, MI = 4) were deeply anesthetized in a bell jar with isoflurane inhalant until a tail or toe pinch produced no reflex movement. Blood was obtained by cardiac puncture, and samples were promptly centrifuged at 10 000 rpm for 15 min at 4°C; the supernatants (plasma) were then transferred into tubes, flash-frozen, and stored at −80°C until processing for reverse phase protein microarray [5].

Sample preparation, printing, scanning, and data analysis for reverse phase protein microarray were performed as described earlier in detail [9]. Primary antibodies were diluted to 10× the optimal Western analysis concentration in antibody incubation buffer and used in the following dilutions: 4-hydroxynonenal (HNE; 1:100) (Calbiochem, 393207), hypoxia-inducible factor-1α (HIF-1α; 1:20) (Santa Cruz, sc-53546), ceruloplasmin (1:20) (GeneTex, GTX28813), vascular endothelial growth factor (VEGF; 1:50) (Abcam, ab53465), von Willebrand factor (vWF; 1:20) (Santa Cruz, sc-8068), neurofilament-heavy chain (NF-H; 1:20) (Sigma–Aldrich, N4142), glial fibrillary acidic protein (GFAP; 1:500) (Abcam, ab7260), myelin basic protein (MBP; 1:20) (Santa Cruz, sc-13914), matrix metalloproteinase 8 (MMP8; 1:20) (Santa Cruz, sc-50384), formyl peptide receptor 1 (FPR1; 1:20) (Santa Cruz, sc-13198), p38 mitogen-activated protein kinase (p38; 1:20) (Cell Signaling Technology, 9212), chemokine (C-C motif) receptor 5 (CCR5; 1:20) (GeneTex, GTX61751), and toll-like receptor 9 (TLR9; 1:100) (Santa Cruz, sc-13218).

Table 1. Oxidative stress and vascular biomarker levels in the plasma at 42 days post injury

Marker	Group	Mean ± SEM	ANOVA		Comparison of means		
			F-value	*p*-value	2–3 (*p*)	4–5 (*p*)	3–5 (*p*)
4-Hydroxynonenal (HNE)	Naïve (1)	4.95 ± 0.17	7.89	0.000	− 0.39	− 0.48	− 0.03
	SS (2)	4.97 ± 0.04			**0.016**	**0.001**	0.999
	SI (3)	**5.37 ± 0.12**					
	MS (4)	4.91 ± 0.07					
	MI (5)	**5.40 ± 0.05**					
Hypoxia-inducible factor-1α (HIF-1α)	Naïve (1)	4.10 ± 0.59	4.53	0.005	− 0.56	− 1.09	− 0.91
	SS (2)	4.11 ± 0.17			0.474	**0.023**	0.137
	SI (3)	4.67 ± 0.18					
	MS (4)	4.48 ± 0.18					
	MI (5)	**5.58 ± 0.41**					
Ceruloplasmin	Naïve (1)	6.29 ± 0.08	10.87	0.000	− 0.41	− 0.51	− 0.06
	SS (2)	6.23 ± 0.05			**0.003**	**0.000**	0.980
	SI (3)	**6.64 ± 0.08**					
	MS (4)	6.20 ± 0.05					
	MI (5)	**6.71 ± 0.12**					
Vascular endothelial growth factor (VEGF)	Naïve (1)	4.29 ± 0.05	6.27	0.001	− 0.32	− 0.59	− 0.21
	SS (2)	4.36 ± 0.07			0.209	**0.001**	0.663
	SI (3)	4.68 ± 0.04					
	MS (4)	4.30 ± 0.06					
	MI (5)	**4.89 ± 0.19**					
von Willebrand Factor (vWF)	Naïve (1)	4.80 ± 0.09	22.74	0.000	− 0.60	− 0.63	− 0.07
	SS (2)	4.74 ± 0.04			**0.000**	**0.000**	0.951
	SI (3)	**5.34 ± 0.06**					
	MS (4)	4.78 ± 0.04					
	MI (5)	**5.41 ± 0.13**					

Mean protein values of naïve, SS, SI, MS and MI rats are log10. Tabulated results include the comparisons for blast injury, SS versus SI (2–3) and MS versus MI (4–5), and for the number of blast events, SI versus MI (3–5). Significant differences in biomarker levels are indicated in boldface.

Slides were incubated with the primary antibody solutions overnight at 4°C, then washed and incubated with the secondary antibodies Alexa Fluor® 633 donkey antisheep (A-21100), Alexa Fluor® 635 goat antimouse (A-31574), Alexa Fluor® 647 goat antirabbit (A-21245), or rabbit antigoat Immunoglobulin G (A-21446) (Molecular Probes®, Invitrogen) at 1:6000 dilution in antibody incubation buffer for 1 h at room temperature. Spot intensity data were imported into a Microsoft Excel-based bioinformatics program for analysis. The total amount of antigen is determined by the *Y*-axis intercept, that is by extrapolating the regression line to zero; reported protein values are log10 [9].

A total of 23 animals (Naïve = 3, SS = 6, SI = 4, MS = 6, MI = 4) were used for the statistical analyses. Student's *t*-test followed by a one-way ANOVA was used to analyze differences in the measured physiological parameters between injured groups and their respective sham groups at baseline, immediately after injury (5 consecutive days for MI rats), and days 1, 3, 7, 14, 26, 36, and 42 post injury. The SI and MI groups were compared on injury day (first injury for MI rats to correspond with SI rats) and each subsequent post injury time point. Statistical significance was reported for blast injury (SS versus SI and MS versys MI*) and for the number of blast events (SI versus MI#). A p value of < 0.05 is depicted by one special character, $p < 0.01$ by two, and $p < 0.001$ by three. Differences in the mean protein biomarker levels measured in plasma were analyzed with ANOVA followed by Tukey's HSD Test. All statistical analyses were performed using IBM SPSS Statistics 20 software. Tests were two tailed using $\alpha = 0.05$; data are presented as the mean ± SEM.

Consistent with our previous findings, the exposure to experimental manipulations alone (i.e. handling, transportation, and anesthesia) can elicit physiological changes as seen in sham animals on the injury day(s) (Fig. 1A–D) [6]. For logistical reasons we were not able to measure the immediate pre and post injury values of the selected physiological parameters, thus the extent and temporal pattern of acute changes remain unknown. However, the restoration of O_2 saturation levels to pre-injury values within a day after a single blast exposure (Fig. 1A) suggests that MI rats similarly recover after each daily exposure (Fig. 1B).

Of the four physiological parameters, only arterial O_2 saturation levels and heart rate changed significantly in response to either type of injury; the detected changes were transient over the length of the experiment (Fig. 1A–D). No significant injury-induced changes (i.e. sham versus injured) were measured in pulse distension and breath rate at any of the time points (data not shown). Importantly, we did not

Table 2. Neuronal, glial, and inflammatory biomarker levels in the plasma at 42 days post injury

Marker	Group	Mean ± SEM	ANOVA		Comparison of means		
			F-value	*p*-value	2–3 (*p*)	4–5 (*p*)	3–5 (*p*)
Neurofilament-heavy chain (NF-H)	Naïve (1)	5.94 ± 0.06	6.29	0.001	−0.37	−0.24	0.06
	SS (2)	5.83 ± 0.05			**0.002**	**0.049**	0.975
	SI (3)	**6.21 ± 0.13**					
	MS (4)	5.90 ± 0.03					
	MI (5)	**6.15 ± 0.04**					
Glial fibrillary acidic protein (GFAP)	Naïve (1)	2.55 ± 0.23	8.87	0.000	−0.98	−1.25	−0.09
	SS (2)	2.94 ± 0.16			**0.038**	**0.000**	0.999
	SI (3)	**3.92 ± 0.39**					
	MS (4)	2.75 ± 0.14					
	MI (5)	**4.01 ± 0.21**					
Myelin basic protein (MBP)	Naïve (1)	4.59 ± 0.14	6.45	0.001	−0.63	−0.7	−0.12
	SS (2)	4.64 ± 0.06			**0.013**	**0.012**	0.984
	SI (3)	**5.28 ± 0.24**					
	MS (4)	4.70 ± 0.07					
	MI (5)	**5.40 ± 0.15**					
Matrix metalloproteinase 8 (MMP8)	Naïve (1)	5.27 ± 0.10	3.24	0.022	−0.12	−0.27	−0.12
	SS (2)	5.25 ± 0.07			0.659	**0.016**	0.720
	SI (3)	5.37 ± 0.04					
	MS (4)	5.21 ± 0.04					
	MI (5)	**5.49 ± 0.07**					
Formyl peptide receptor 1 (FPR1)	Naïve (1)	4.96 ± 0.07	4.02	0.009	−0.33	−0.44	−0.06
	SS (2)	5.00 ± 0.06			0.175	**0.034**	0.996
	SI (3)	5.34 ± 0.04					
	MS (4)	4.95 ± 0.05					
	MI (5)	**5.39 ± 0.09**					
P38 mitogen-activated protein kinase (p38)	Naïve (1)	3.10 ± 0.22	5.26	0.002	−0.56	−0.73	−0.02
	SS (2)	2.97 ± 0.05			0.091	**0.008**	0.999
	SI (3)	3.52 ± 0.15					
	MS (4)	2.80 ± 0.13					
	MI (5)	**3.53 ± 0.12**					
Chemokine (C-C motif) receptor 5 (CCR5)	Naïve (1)	2.56 ± 0.12	5.08	0.003	−0.85	−0.48	0.41
	SS (2)	3.05 ± 0.10			**0.041**	0.33	0.697
	SI (3)	**3.90 ± 0.11**					
	MS (4)	3.01 ± 0.12					
	MI (5)	3.50 ± 0.07					
Toll-like receptor 9 (TLR9)	Naïve (1)	4.00 ± 0.06	0.82	0.523	−0.29	0.53	−0.08
	SS (2)	4.01 ± 0.39			0.987	0.876	0.999
	SI (3)	4.30 ± 0.17					
	MS (4)	3.95 ± 0.74					
	MI (5)	4.38 ± 0.48					

Mean protein values of naïve, SS, SI, MS and MI rats are log10. Tabulated results include the comparisons for blast injury, SS versus SI (2–3) and MS versus MI (4–5), and for the number of blast events, SI versus MI (3–5). Significant differences in biomarker levels are indicated in boldface.

detect any lasting changes between SI and MI animals in any of the measured vitals.

Forty-two days post injury, it appears that repeated exposure to mild blast overpressure resulted in hypoxia and oxidative stress as reflected in significantly increased plasma levels of oxidative stress markers HNE, HIF-1α, and ceruloplasmin (Table 1). At this late time point, HNE and ceruloplasmin levels were also increased in SI animals albeit to a lesser degree than in MI animals. These changes indicate a potential role for hypoxia and oxidative stress in the pathobiology of blast-induced TBI [10]. An increase in HNE levels during periods of oxidative stress is due to an increase in the lipid peroxidation chain reaction that affects a variety of biological pathways, including the cell cycle and cellular adhesion. Elevated ceruloplasmin levels are another indication of oxidative stress triggered by hypoxia [11]. As well demonstrated in stroke models, hypoxia can cause lasting increases in HIF-1α levels [12]. HIF-1α plays a crucial role in the adaptive and restorative response of organisms following neuronal insults (e.g. stroke and TBI) as it coordinates the expression of numerous genes to cope with noxious conditions thus mitigating the effects of ischemic conditions.

TBI also adversely affects several vascular functions including blood brain barrier permeability [13]. VEGF along with vWF is a key regulator of vascular permeability and other endothelial functions [14, 15]. The more robust increase in VEGF levels in MI rats suggests that VEGF may be involved in mediating the cumulative, more severe outcomes of rmTBI (Table 1). While VEGF levels were only significantly elevated at 42 days post injury in MI rats, vWF plasma levels remained significantly elevated in response to both types of injury. These findings implicate long-term alterations in endothelial functions after TBI including increased blood brain barrier permeability, which enables large molecules such as neuron- and glia-specific proteins to cross into the systemic circulation.

Consistent with our previous findings, even a single mild blast exposure significantly increased NF-H, GFAP, and MBP levels in the plasma (Table 2). Unlike the neuronal marker NF-H, GFAP, and MBP concentrations were relatively higher in MI animals than in SI animals as observed in the majority of the tested protein markers. Increased NF-H and MBP concentrations reflect damage to axons and their myelin sheaths as a result of the physical forces of the blast. Damage to axons and white matter tracts have been identified both clinically and experimentally as hallmarks of blast TBI along with damage to astroglia reflected in increased GFAP levels [16, 17].

Of the markers associated with various aspects of inflammation, MMP8, FPR1, and p38 were only elevated in MI animals suggesting greater damage accumulation and/or a more severe outcome in rmTBI. Given the role of these markers in the mediation of the neuroinflammatory process in several central nervous system disorders [18], it is not surprising that rmTBIs increase the risk for debilitating conditions like chronic traumatic encephalopathy. At this late time point, CCR5 levels were significantly elevated in SI animals alone while TLR9 was relatively unchanged in both injured groups. A potential explanation for the insignificant CCR5 response in MI rats is the late sampling time after injury. This can also account for the insignificant TLR9 levels in both injured groups due to the protein's rapid elevation and decline after injury.

In conclusion, the exposure to single and repeated mild blast at this frequency of insults triggers lasting changes in the form of oxidative stress, vascular abnormalities, and neuronal and glial cell damage/death. The chronic nature of these changes is particularly important considering that a rat week is the equivalent of 6–8 human months. However, we found no increase in the magnitude of the cumulative effect at this late time point suggesting that the frequency of the repetitive insults plays a critical role in determining the extent of the damage accumulation in rmTBI.

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The authors have declared no conflict of interest.

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Diffusion Tensor Imaging Reveals Acute Subcortical Changes after Mild Blast-Induced Traumatic Brain Injury

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Mild blast-induced traumatic brain injury (mbTBI) poses special diagnostic challenges due to its overlapping symptomatology with other neuropsychiatric conditions and the lack of objective outcome measures. Diffusion tensor imaging (DTI) can potentially provide clinically relevant information toward a differential diagnosis. In this study, we aimed to determine if single and repeated (5 total; administered on consecutive days) mild blast overpressure exposure results in detectable structural changes in the brain, especially in the hippocampus. Fixed rat brains were analyzed by ex vivo DTI at 2 h and 42 days after blast (or sham) exposure(s). An anatomy-based region of interest analysis revealed significant interactions in axial and radial diffusivity in a number of subcortical structures at 2 h only. Differences between single- and multiple-injured rats were largely in the thalamus but not the hippocampus. Our findings demonstrate the value and the limitations of DTI in providing a better understanding of mbTBI pathobiology.

Mild traumatic brain injury (mTBI) continues to be the least understood form of traumatic brain injury (TBI) despite its high incidence and substantial toll on patients and health care systems[1]. In the military, mTBIs are mostly caused by the exposure to low levels of blast from improvised explosive devices resulting in mild blast-induced TBI (mbTBI)[2–4]. The diagnosis of mbTBI currently relies on subjective assessments and self-reports of symptoms such as disorientation, altered states of consciousness, headaches, and emotional and cognitive dysfunction—all of which are involved in post-traumatic stress disorder (PTSD)[5]. Because of the mild and transient nature of symptoms that follow mbTBI, soldiers typically return to duty and are frequently re-exposed to additional mild blasts. Studies have suggested that repeated mbTBI is a risk factor for developing late onset neurodegenerative conditions such as chronic traumatic encephalopathy (CTE)[6].

Objective outcome measures can provide especially valuable, clinically relevant information in a non-/minimally invasive and repeatable manner. Various modalities of magnetic resonance imaging (MRI), including diffusion tensor imaging (DTI), have been utilized in clinical settings following TBI[7–10]. However, only a limited number of clinical studies included readouts at several post-injury time points in Veterans[11–17]. DTI's sensitivity relative to conventional imaging tools has prompted its recent use in experimental mTBI[18–20] with a few rodent blast-induced TBI (bTBI) studies[21–24]. These studies identified a number of brain regions, including the hippocampus and the cerebellum, as being affected in mbTBI[25]. Injury-induced changes in serum, cerebrospinal fluid, and tissue protein biomarker levels have also been extensively investigated in both clinical and experimental TBI[26–28]. Together, imaging and molecular biomarkers would enable the monitoring of pathological processes over time and allow for more direct comparisons between experimental findings and clinical TBI cases.

The full potential and limitations of using imaging and molecular biomarkers in the diagnosis and monitoring of TBIs, especially mTBIs, are currently unknown due to a substantial gap between clinical and experimental findings and their translatability[29]. Furthermore, our understanding of how structural changes relate to cellular, molecular, and functional changes in TBI is very limited. Our previous works using the rodent model of single and repeated mbTBI recapitulated some of the behavioral changes that are observed in human bTBI[30]. Using histo-

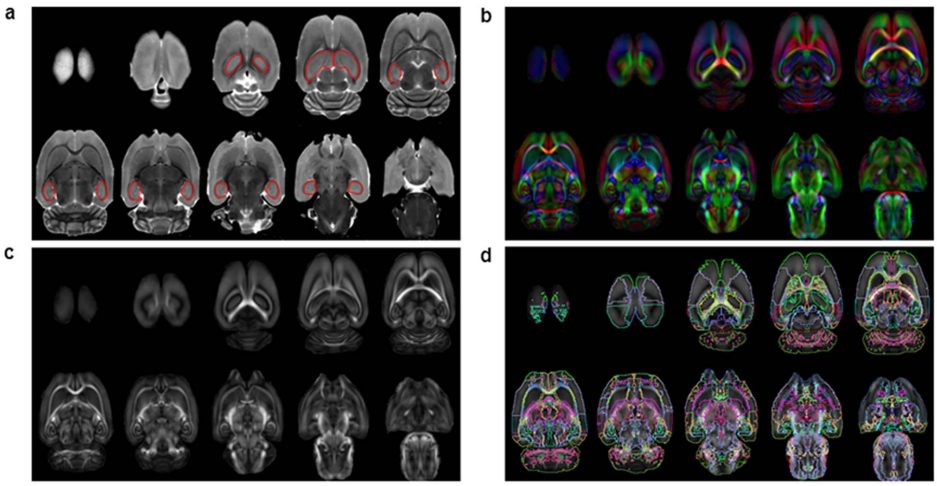

Figure 1 | MRI data analysis. (a) A T2-weighted image from a single subject with the hippocampus manually outlined in red. (b) A mean directionally encoded color image. (c) Map of FA derived from DTI of all spatially registered brains (every second slice is shown). (d) The registered anatomical ROIs derived from the atlas overlaid on the FA map for visualization.

logic and proteomic analyses of functionally relevant brain regions and peripheral blood, we identified several pathologies at different post-injury time points. These include neuronal and glial damage and/or death, axonal damage, metabolic and vascular changes, and inflammation. Additionally, we identified several pathologies that include neuronal and glial damage and/or death, axonal damage, metabolic and vascular changes, and inflammation at different post-injury time points using histologic and proteomic analyses of functionally relevant brain regions and peripheral blood[31–33]. In this preliminary imaging study, we aimed to determine if the same exposure to single and repeated mild blast overpressure that resulted in the abovementioned changes also induced structural changes that are detectable by DTI.

Results

We selected two of our previously tested post-injury termination time points, 2 h and 42 days, for the DTI analyses to mimic early and delayed clinical interventions. A manual region of interest (ROI) analysis was first used to assess hippocampal volume and fractional anisotropy (FA) in the hippocampus as shown in Fig. 1a. No significant differences were identified in hippocampal volume or FA values at either time point (Fig. 2). An anatomically defined ROI analysis was then performed as shown in Fig. 1b–d. In rats terminated ~2 h after blast (or sham) exposure(s), no brain regions had a significant interaction for FA. However, axial diffusivity (AD) and radial diffusivity (RD) had significant interactions in regions of the stria terminalis, thalamic subregions, and the cerebellum. Post hoc analysis revealed that the single-injured (SI) and multiple-injured (MI) groups were significantly different from one another largely in the thalamus and thalamic nuclei. Regions exhibiting significant blast event-related differences (i.e., single vs. repeated blast) are shown in Fig. 3 and Table 1; mean DTI values for these regions are provided in Fig. 4. No brain regions exhibited significant ROI changes in rats terminated 42 days after blast (or sham) exposure(s).

Discussion

Elucidating the role of repeated mbTBI in the development of neurodegenerative conditions is a pressing issue for the military health care system. To that end, a better understanding of mbTBI pathobiology, the period of cerebral vulnerability between insults, and the synergistic effect of repeated injury is critical. In conducting a series of studies comparing single and repeated mild blast injury (5 overpressure exposures administered on consecutive days), we aimed to assess the extent of the damage accumulation in mbTBI (i.e., the

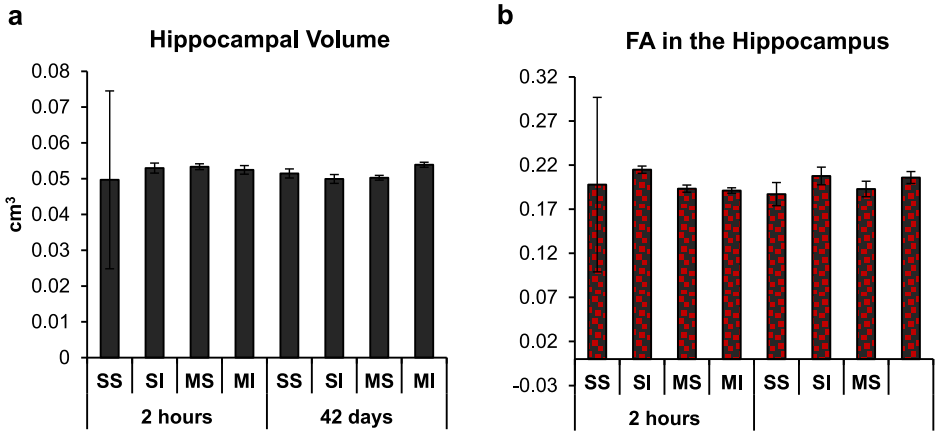

Figure 2 | Volumetric and DTI measures in the hippocampus. (a) Hippocampal volume (cm^3) of sham (SS, single sham; MS, multiple sham) and injured (SI, single-injured; MI, multiple-injured) rats terminated at 2 h and 45 days after blast (or sham) exposure(s). (b) Fractional anisotropy (FA) in the hippocampi of rats at the same time points. Data are presented as the mean ± SEM.

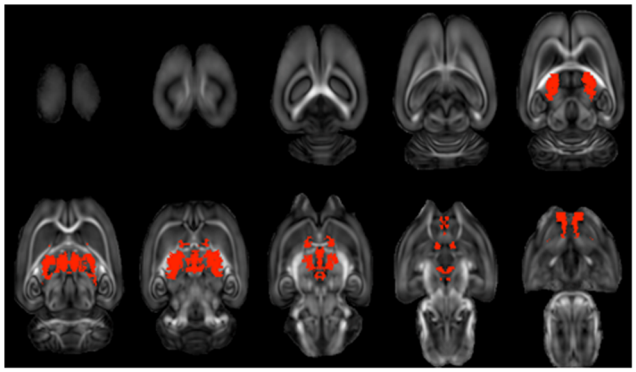

Figure 3 | Brain regions exhibiting significant ROI changes. Regions with a significant Blast x No. of Events interaction were first identified; those with significant differences between single-injured and multiple-injured rats (corrected for multiple comparisons) are shown in red.

cumulative effect of the injury) at different post-injury time points. Of particular interest to us is correlating cellular and molecular level changes with structural and neurocognitive changes toward a definitive diagnosis for mbTBI. The objective of this study was to determine if the exposure to single and repeated mild blast overpressure, which resulted in significant functional, cellular, and molecular changes, also induced structural changes that are detectable by ex vivo DTI.

Based on a number of bTBI studies that implicate the hippocampus in the development of neurobehavioral symptoms, we expected to detect injury-induced structural and/or volumetric changes in this region due to its involvement in TBI[34,35]. We previously found significantly increased numbers of apoptotic, TUNEL-positive cells in the hilus and granular cell layer of the hippocampus as early as 2 h post-injury in both single- and multiple-injured rats[36]. However, we found no significant changes in hippocampal volume or FA in the hippocampus in our current study. This discrepancy may be related to the current spatial resolution of DTI. Another plausible explanation is that even though we found significantly increased rates of cell death in the hippocampus, we also found a substantive gliotic response[30,31,33,37]. Such astroglial hypertrophy can potentially compensate for the loss in volume caused by cell death.

A recent ex vivo DTI rodent study has shown that the microstructure of the hippocampus can be significantly affected in mbTBI[23]. Consistent with impaired cognitive performance, FA values were significantly decreased in select brain regions of blast-exposed rats relative to their sham controls at 4 and 30 days post-injury. The affected brain regions included the hippocampus, thalamus, and brainstem. It is important to note, however, that the blast model and experimental design of our and the Budde et al. study are very different. Nonetheless, hippocampal abnormalities have been found in a number of clinical mbTBI studies using various imaging modalities[12,15,17,38–40].

Since no significant hippocampal changes were detected in our work, an automated, anatomical ROI analysis without a priori assumptions of affected regions was used to examine the brains[23]. Compared to a voxel by voxel approach that includes thousands of independent statistical tests, the whole brain anatomical ROI approach reduces the number of statistical comparisons but avoids tedious manual definition of brain regions[41]. The results of this analysis demonstrated significant changes that are largely confined to midline thalamic structures and the cerebellum. Post hoc analysis revealed that SI and MI rats were significantly different from one another in the thalamus and thalamic nuclei. Previous bTBI studies also found changes in the thalamus using DTI[23] and histological methods[42]. Thalamus-mediated functions account for a significant number of the most frequently reported neurobehavioral symptoms in clinical mbTBI. Among the leading complaints are sleep and emotional disturbances as well as altered sensory sensitivities, both auditory and visual[14].

Cerebellar abnormalities have been found in most human bTBI imaging studies[12,15,16,43] and in a recent rodent bTBI study[24]. These findings illustrate the region-specific vulnerability of the brain to different types of physical insults—an important albeit poorly understood issue in TBI. The cerebellum's susceptibility to injury maybe due to its anatomy; it is located in a relatively small sub-compartment of the skull and the ratio between cerebellar white and grey matters is different from that in the cerebrum. Primary blast injury mainly exerts damage at the interface of biological materials with differing physiochemical properties (e.g., grey and white matter). Indeed, white matter damage—including cerebellar white matter—has been found in virtually all human bTBI imaging studies. Functionally, the cerebellum is involved in certain cognitive and learning functions,

Table 1 | Brain regions exhibiting significant blast event-related effects at 2 h post-injury

	Fractional Anisotropy		Axial Diffusivity				Radial Diffusivity			
	Blast x No. of Events Interaction		Blast x No. of Events Interaction		SI vs. MI *t*-Test		Blast x No. of Events Interaction		SI vs. MI *t*-Test	
Brain Region	*F* value	*p* value[a]	*F* value	*p* value[b]	*t* value	*p* value[b]	*F* value	*p* value[b]	*t* value	*p* value[b]
Stria Terminalis	4.55	0.065	19.28	0.017	6.34	**0.023**	11.91	0.068	4.80	0.099
Posterior Hypothalamic Nucleus	1.25	0.296	13.56	0.043	3.78	0.145	13.57	0.048	4.06	0.189
Islands of Calleja	1.95	0.200	26.50	0.006	−9.89	**0.004**	34.55	0.003	−6.23	**0.042**
Olfactory Tubercle	1.02	0.341	35.79	0.002	−5.28	**0.048**	70.63	0.000	−7.95	**0.010**
Ventral Nucleus of Thalamus	0.98	0.352	29.78	0.004	9.02	**0.007**	20.89	0.014	6.81	**0.026**
Lateral Dorsal Nucleus of Thalamus	3.60	0.094	15.33	0.032	7.01	**0.017**	18.44	0.021	7.28	**0.021**
Lateral Posterior Nucleus of Thalamus	2.06	0.189	15.96	0.026	5.55	**0.040**	18.47	0.018	5.65	0.059
Central Lateral Nucleus of Thalamus	3.09	0.117	20.11	0.014	7.18	**0.014**	24.46	0.009	8.05	**0.005**
Medial Dorsal Thalamus	1.93	0.203	15.71	0.029	7.74	**0.011**	17.07	0.026	7.31	**0.016**
Midline Thalamic Nuclei	7.55	0.025	21.27	0.011	10.00	**0.002**	15.91	0.031	6.72	**0.031**
Thalamus	0.35	0.568	13.29	0.047	4.85	0.065	13.89	0.044	4.77	0.108
Cerebellum	4.24	0.073	17.26	0.022	−3.41	0.212	21.95	0.011	−5.05	0.089

[a]uncorrected.
[b]false discovery rate corrected.
SI, single-injured ($n = 3$); MI, multiple-injured ($n = 3$).
Statistically significant differences between SI and MI rats are indicated in boldface.

hence the detected changes are consistent with clinically observed abnormalities[44,45].

Among the other affected brain structures is the stria terminalis, which serves as a major relay site within the hypothalamic-pituitary-adrenal axis[46]. Similar changes were also found in the olfactory tubercule, including the islands of Calleja. The olfactory tubercule has been shown to play a role in behavioral response as it is interconnected with several brain regions with sensory and arousal/reward functions[47]. In fact, injury to the islands as a result of restricted blood flow has been linked to a number of behavioral and emotional responses such as amnesia and changes in personality—behavioral changes that are not possible to assess in animal models.

A critical limitation toward better understanding human mbTBI is inherent variability as well as the unknown biophysical forces that are experienced during injury. Additionally, most existing DTI studies of veterans have been performed years after the injury. Animal models of mbTBI allow for direct testing of the many effects of blast wave characteristics under carefully controlled conditions[48]. However, we currently have no clear understanding of how human years (physiologically and pathologically speaking) translate into rat months (or weeks). Furthermore, the lack of a consensus regarding a high fidelity experimental bTBI model—as demonstrated by the imaging findings obtained using various blast models—is a major impediment to studying the physical and biological effects of primary blast injury.

Another pressing issue is how DTI findings in mbTBI (or any other neurological disorder) relate to changes detectable by proteomics or histology. We emphasize this point because although rats terminated at 42 days did not exhibit significant ROI changes as measured by DTI, proteomic analyses of plasma at the same time point showed significant and persistent molecular pathologies in SI as well as MI rats[36,49]. These include inflammation, metabolic and vascular changes, neuronal and glial cell damage and/or death, and axonal damage.

A technical limitation of our study is the use of fixed tissues in ex vivo DTI, mainly due to altered diffusivity of water molecules. Nonetheless, previous studies have demonstrated that ex vivo DTI provides valuable structural information that correlates with in vivo changes albeit to a varying extent. This may partially account for the poor correlation between cellular changes obtained by conventional histology and volumetric/DTI measures in the hippocampus. It should be noted that animal in vivo imaging has its own issues with scanning times (and corresponding anesthesia times), image acquisition protocols, and motion artifacts being the major ones.

Despite the increased attention in recent years on blast as a mechanism of mTBI, the subject of how blast waves affect the brain along with diagnosing mbTBI are still a matter of considerable debate. The abovementioned caveats underline the importance of combining objective and clinically relevant outcome measures in experimental TBI to validate and correlate findings, to enable more direct comparisons of pathologies observed in animal and in clinical TBI research, and to enable the development of sensitive and specific diagnostics for mbTBI[29].

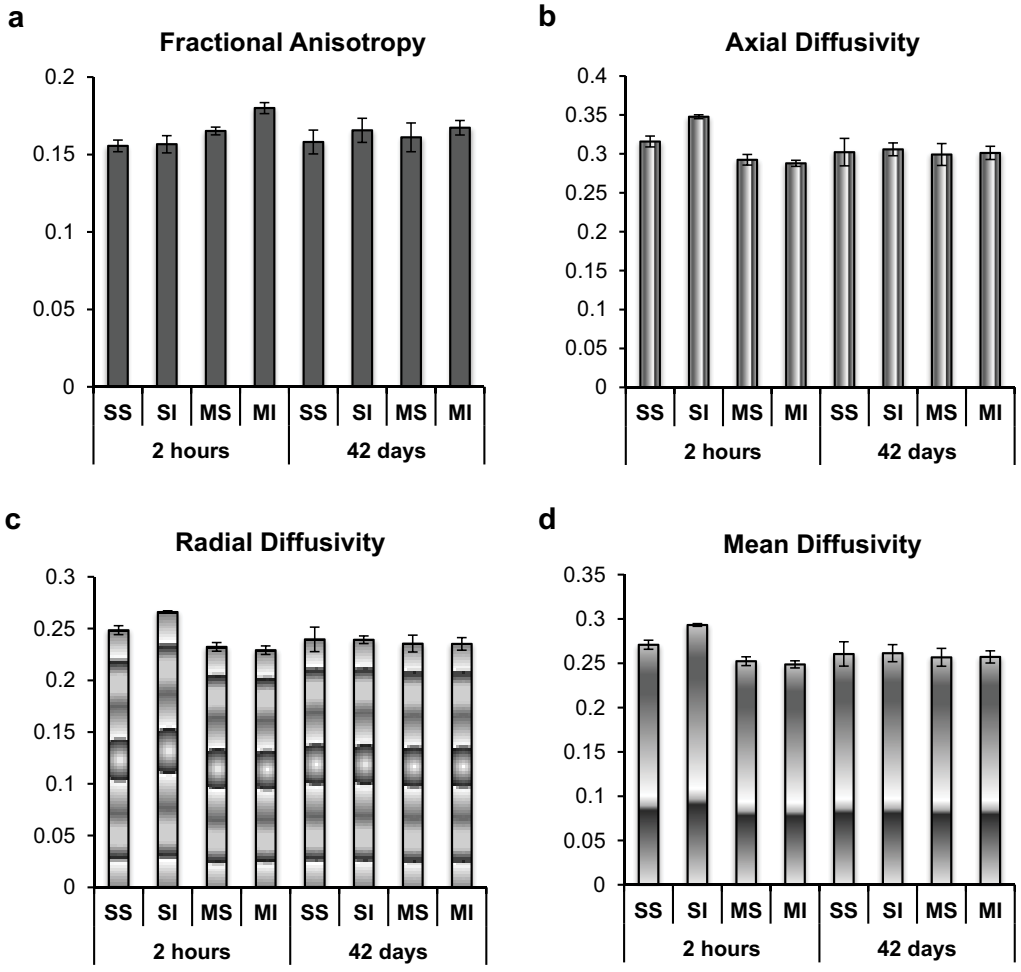

Figure 4 | Mean DTI values at the two time points. Data were extracted from each subject as a single value from the ROIs showing significance in the anatomical ROI analysis. Data are presented as the mean ± SEM for each experimental group (SS, single sham; SI, single-injured; MS, multiple sham; MI, multiple-injured).

Methods

Animals and housing conditions. A total of 60 male Sprague Dawley rats (weight at arrival: 245–265 g) (Charles River Laboratories, Wilmington, MA) were used in the original experiments[36,49]. All animals were housed in standard rat cages with a built-in filter in a reverse 12-h light 12-h dark cycle with food and water ad lib. Animals were handled according to protocol approved by the Institutional Animal Care and Use Committee at the Uniformed Services University (USU; Bethesda, MD).

Experimental groups and manipulations. All animals underwent a 5 day acclimation and handling period and were later assigned to the following groups: naïve, single sham (SS), single-injured (SI), multiple sham (MS), and multiple-injured (MI) as described earlier[36,49]. Rat numbers in the early and late termination groups were: ($N = 30$; naïve = 3, SS = 6, SI = 7, MS = 6, MI = 8) and ($N = 30$; naïve = 3, SS = 6, SI = 7, MS = 6, MI = 8), respectively. Naïve rats were kept in the animal facility at USU without any manipulation for the duration of the studies. SS rats were transported once from USU to Walter Reed Army Institute of Research (Silver Spring, MD) and anesthetized in an induction chamber with a 4% isoflurane (Forane; Baxter Healthcare Corporation, Deerfield, IL) in air mixture delivered at 2 L/min for 6 min. MS rats were similarly transported and anesthetized once per day for 5 consecutive days. SI and MI rats underwent the same procedures as their respective sham controls in addition to receiving a single or multiple (5 total) mild blast exposure(s)[36,49].

Injury conditions. Anesthetized rats in chest protection (weight at injury: 300–330 g) were placed in the shock tube holder in a transverse prone position with the right side facing the direction of the membrane and the incidence of the blast waves. Blast overpressure was generated using a compressed air-driven shock tube yielding a single blast overpressure wave (average peak total pressure: ~137 kPa at the animal level) to produce a mild injury as described in detail[31,37,50]. Following blast (or equivalent time spent anesthetized as a sham), animals were moved to an adjacent bench top for observation and then transported back to the USU animal facility at the conclusion of each injury day.

Preparation of specimens for imaging. A subset of animals from each experiment [(2 h termination: $n = 11$; SS = 2, SI = 3, MS = 3, MI = 3) and (42 day termination: $n = 16$; SS = 4, SI = 4, MS = 4, MI = 4)] was used for MRI/DTI analyses; all other animals were used for proteomics as described earlier[36,49]. Rats were deeply anesthetized with isoflurane inhalant until a tail pinch produced no reflex movement, then transcardially perfused with cold phosphate-buffered saline (PBS) followed by a 4% paraformaldehyde in 1x PBS solution. The brains were removed and post-fixed in the same solution overnight at 4°C and then transferred to a 1x PBS solution containing 0.1% sodium azide until scanning. No hemorrhage or any other signs of macroscopic damage were detected in any of the animals.

Image acquisition. Fixed brains underwent ex vivo DTI within 2 days of perfusion fixation on a Bruker 7 T vertical bore system. Brains were immersed in susceptibility-matching fluid (Fomblin; Solvay Solexis, Inc., West Deptford, NJ) and inserted into a radiofrequency coil 3 cm in diameter. A three-echo diffusion-weighted spin echo sequence was employed (TR = 4 s; TE = 20 ms (first echo; 7.5 ms echo spacing) to acquire diffusion-weighted images (b = 1200 s/mm^2) along 30 directions[51] with diffusion gradient duration (δ) and separation (Δ) of 4 and 10 ms, respectively, along with 5 non diffusion-weighted images[52]. The slice thickness was 0.5 mm with an in-plane resolution of 0.234 mm^2 and a 30 mm^2 field of view (128^2 matrix). The full experiment required 6 h of continuous imaging. DTI data was reconstructed using a linear least squares fit to derive parameter maps of FA, AD, and RD using custom Matlab routines[53].

Data analysis. The analysis of MRI data included volumetric and DTI measures in the hippocampus and an anatomical ROI analysis of DTI data without a priori assumptions of affected regions. Hippocampal volume and FA in the hippocampus were derived from manual segmentation of the hippocampus on T2-weighted and FA maps, respectively, by an operator blinded to animal conditions. For unbiased quantification of DTI measures using anatomically based ROIs, DTI volumes from all subjects were first registered to a common space using an iterative, tensor-based registration routine implemented in DTI-TK[54]. Rigid-body, affine, and diffeomorphic (piecewise affine) methods were used in succession to progressively improve registration accuracy, as this approach has been shown to be superior to other routines[55]. The final image resolution was 120 × 120 × 500 μm^3. Anatomical ROIs were derived from a digital rat brain atlas included as part of the Medical Image Visualization and Analysis Software (MIVA) software package[56]. The regions consisted of 87 subregions of the brain initially derived from the Paxinos Rat brain atlas[57]. A mask of white matter regions derived from the atlas was registered to a mask of white matter regions derived from DTI by thresholding the FA maps at 0.2. An FA value of 0.2 was chosen empirically since it effectively masked the white matter tracts. It should be noted that this threshold value was used for the registration of the ROIs, not for quantification. Registration employed a point-set based registration metric incorporated into Advanced Normalization Tools (ANTS) software package, including elastic warping[58]. The resulting overlap demonstrated high correspondence between the DTI and atlas-based white matter structures (Fig. 1C). The mean FA, AD, and RD within each ROI were derived from each of the registered DTI volumes from each subject for subsequent statistical analysis (Fig. 4).

Statistical analysis. Twenty-seven animals were used for the analyses (2 h termination, $N = 11$; 42 day termination, $N = 16$). A mixed-effect ANOVA was first performed to identify any significant effects of left/right (L/R) asymmetry. Since none of the brain regions exhibited a significant Blast x No. of Events x Side (L/R) interaction, the effect of side was collapsed for all subsequent analyses. For hippocampal volume and FA in the hippocampus, ANOVAs followed by Tukey's HSD test were performed separately at each time point. Subsequently, a one-way ANOVA was performed for each condition across the two time points.

For DTI, a two-way ANOVA was performed to compare the main effects of Blast x No. of Events interaction. Regions that exhibited a significant interaction were subjected to post-hoc analysis using a Student's t-test to compare the SI and MI groups. All statistical tests were corrected for multiple comparisons (87 individual ROIs) by controlling for the false discovery rate[59]. A Spearman correlation analysis was used to identify brain regions significantly correlated to either the number of blast events or the number of sham events. A corrected p value of 0.05 was considered significant for all tests.

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Author contributions

A.K. and E.K. carried out animal studies, including the preparation of specimens for imaging. J.L. designed and supervised blast overpressure exposures at Walter Reed. M.B. performed and analyzed MRI/DTI measures under J.F.'s supervision at the NIH. A.K., M.B., and D.A. wrote the main manuscript text; A.K. and M.B. generated and formatted figures 1–4 and table 1. A.K. and D.A. reviewed the manuscript prior to submission.

Additional information

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